# BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

# IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.**

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01R 33/46 — A1

(11) International Publication Number: WO 94/20863

(43) International Publication Date: 15 September 1994 (15.09.94)

(21) International Application Number: PCT/GB94/00425

(22) International Filing Date: 4 March 1994 (04.03.94)

(30) Priority Data:
9304372.7 4 March 1993 (04.03.93) GB

(71) Applicant *(for all designated States except US)*: BIRKBECK COLLEGE [GB/GB]; Malet Street, London WC1E 7HX (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SADLER, Peter, John [GB/GB]; 40 Albion Road, Pitstone, Leighton Buzzard, Bedfordshire LU7 9AY (GB). VILES, John, Howard [GB/GB]; 39 Oakfield Avenue, Hertfordshire SG3 6NP (GB). TUCKER, Alan [GB/GB]; 14 Tudor Close, Hatfield, Hertfordshire AL10 9EJ (GB). PATEL, Sunil, Unka [GB/GB]; 3 Marmion Grove, Scotts Green, Dudley, West Midlands DY1 2DG (GB).

(74) Agent: MARCH, Gary, Clifford; Batchellor, Kirk & Co., 2 Pear Tree Court, Farringdon Road, London EC1R ODS (GB).

(81) Designated States: AU, CA, FI, GB, JP, NO, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
*With international search report.*

(54) Title: DETECTION OF ALBUMIN IN PHYSIOLOGICAL FLUIDS

(57) Abstract

A method of detecting albumin or one or more amino acid residues thereof, in a physiological fluid containing albumin and other proteins, and optionally free amino acids, which comprises subjecting a sample of the fluid to $^1$H NMR spectroscopy which is single pulse or 2 dimensional spectroscopy, in which the resolution is enhanced.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| **AT** | Austria | **GB** | United Kingdom | **MR** | Mauritania |
| **AU** | Australia | **GE** | Georgia | **MW** | Malawi |
| **BB** | Barbados | **GN** | Guinea | **NE** | Niger |
| **BE** | Belgium | **GR** | Greece | **NL** | Netherlands |
| **BF** | Burkina Faso | **HU** | Hungary | **NO** | Norway |
| **BG** | Bulgaria | **IE** | Ireland | **NZ** | New Zealand |
| **BJ** | Benin | **IT** | Italy | **PL** | Poland |
| **BR** | Brazil | **JP** | Japan | **PT** | Portugal |
| **BY** | Belarus | **KE** | Kenya | **RO** | Romania |
| **CA** | Canada | **KG** | Kyrgyzstan | **RU** | Russian Federation |
| **CF** | Central African Republic | **KP** | Democratic People's Republic of Korea | **SD** | Sudan |
| **CG** | Congo | | | **SE** | Sweden |
| **CH** | Switzerland | **KR** | Republic of Korea | **SI** | Slovenia |
| **CI** | Côte d'Ivoire | **KZ** | Kazakhstan | **SK** | Slovakia |
| **CM** | Cameroon | **LI** | Liechtenstein | **SN** | Senegal |
| **CN** | China | **LK** | Sri Lanka | **TD** | Chad |
| **CS** | Czechoslovakia | **LU** | Luxembourg | **TG** | Togo |
| **CZ** | Czech Republic | **LV** | Latvia | **TJ** | Tajikistan |
| **DE** | Germany | **MC** | Monaco | **TT** | Trinidad and Tobago |
| **DK** | Denmark | **MD** | Republic of Moldova | **UA** | Ukraine |
| **ES** | Spain | **MG** | Madagascar | **US** | United States of America |
| **FI** | Finland | **ML** | Mali | **UZ** | Uzbekistan |
| **FR** | France | **MN** | Mongolia | **VN** | Viet Nam |
| **GA** | Gabon | | | | |

# DETECTION OF ALBUMIN IN PHYSIOLOGICAL FLUIDS

This invention relates to the detection of the protein albumin in physiological body fluids which can be of human or other animal origin, such as blood plasma or serum, using nuclear magnetic resonance spectroscopy.

Although it is known that proton $^1$H-NMR spectroscopy can be used to detect constituents of body fluids such as blood plasma it is generally accepted that no $^1$H-NMR resonances can be obtained with such techniques about the protein albumin. No resonances have been specifically assigned to amino acid residues of plasma proteins - they are broad and routinely filtered out.

The present invention is concerned with the novel application of resolution enhancement to $^1$H NMR spectra of blood plasma or sera which allows protons of individual amino acid residues to be detected in samples of intact sera or plasma. This can be combined with the optional addition of a reagent (such as $Ni^{2+}$ ion donor) which enables confirmation of albumin resonance assignments.

Albumin is a very large protein (66.5 kDa, 585 amino acids) and plays a major part in the transport of drugs around the body, and it may be abnormally affected in various clinical states. It is the major protein present in blood and its concentration, activity and physical state may be affected in a range of diseased states. A technique particularly for quantitative determination of albumin in intact human or animal sera is highly desirable as a diagnostic aid for monitoring physical and/or chemical changes in the nature of a person's serum albumin associated with diseased states, drug transport, and metal-binding events as several practical applications of the present method.

Albumin undergoes significant pH-dependant structural transitions and the present methods may be of

value in monitoring variations in such transitions attributable to clinical conditions or drug delivery or transport mechanisms. The reader is referred to, for example, Eur. J. Biochem. 212, 811-817 (1993) for details of such transitions at the N-terminus of the albumin protein molecule.

The fluid examined can be for example blood plasma or serum prepared by well-known methods. In the case of plasma, a range of anticoagulants can be used including calcium chelating agents (e.g. ethylenediamine tetraacetic acid and salts thereof - EDTA, citrate). The present invention can resolve a fraction of the total albumin resonances, but it is not necessary to purify or separate the albumin from the physiological fluid used for the investigation.

According to this invention we provide a method of detecting albumin or one or more amino acid residues thereof, in a physiological fluid containing albumin and other proteins, and optionally free amino acids, which comprises subjecting a sample of the fluid to $^1H$ NMR spectroscopy which is single pulse or 2 dimensional spectroscopy, in which the resolution is enhanced.

In order to quantify the amount of albumin present in a given sample, reference can be made to standard samples and standard additions. Identity of peaks in the NMR spectra obtained from a sample of physiological fluid can be ascertained by reference to published data on isolated albumin and the reader is referred to, for example, Eur. J. Biochem. 205, 631-643 (1992) for the assignment of spin systems in proton NMR studies of bovine serum albumin.

The area under the peaks can be integrated or the peak height can be used as a measure of the quantity of albumin present. Comparisons with known data can be made to complete the quantitative determination.

Typically 0.5 ml of plasma or serum is placed in a

5 mm glass NMR tube. A small amount of a deuteriated solution (e.g. $D_2O$,5% v/v) can be added if desired to provide a lock signal for stabilisation of the magnetic field.

High frequency spectrometers (e.g. 500 or 600 MHz) provide the best separation of resonances, and are preferred but other frequencies can be used.

Resolution enhancement of NMR spectra is well developed and known. In the present method, apodization may be used to selectively filter broad peaks. It is preferred to use a combination of (unshifted) sine-bell and exponential functions, with a line broadening of about 2Hz. 2D-TOCSY (totally correlated spectroscopy) $^1$H-NMR has been found to be particularly useful in identifying specific amino acid residues and is therefore preferred.

It is known that albumin has specific metal binding sites for copper, nickel, calcium, cadmium, zinc, gold and mercury. These metals, as ions in solution, bind to and thereby form a complex with albumin. We have found that using metal-albumin complexes and preferably the nickel-albumin complex can improve the technique considerably as the resonances shift slightly whilst still affording sharp resolution. Although nickel may be used as a complex-forming reagent to aid investigations, it is possible to use other metals such as gold which bind to a different part of the albumin molecule, for probing other amino acid residues in the protein.

If it is desired to use a metal reagent, nickel can be added to the sample of physiological fluid as a simple salt (e.g. nickel chloride) or as a complex with specific ligands including chelating agents which bind $Ni^{2+}$ less strongly than albumin. An appropriate time should be allowed for transfer of $Ni^{2+}$ to albumin. Optimum albumin detection is usually obtained when the $Ni^{2+}$ : albumin molar ratio is 1:1 or less than 1:1.

Accordingly the skilled worker subjecting samples to the NMR technique may obtain well resolved signals and specific signals can be shifted to another part of the spectrum but nevertheless remain sharp. Those signals can then be related to a particular part of the albumin molecule.

Resonances of albumin can be recognised by the specific shifts in the presence of the reagent. Abnormal albumins may be recognised by the abnormal behaviour of their resonances in the presence of the reagent.

For the most effective measurements and monitoring using the present $^{1}$H-NMR techniques the serum or plasma should be removed from intact blood and then subjected to the method. If water is present, its resonance should be suppressed.

A particular feature of this invention is that even individual amino acid residues of the albumin protein can be detected within the physiological fluid without separation of the albumin and without damage thereto, for example Aspl, Ala2 and His3 of human serum albumin (HSA). The capacity of the present method to probe, detect and quantify individual 'parts' of the albumin protein within a given sample of physiological fluid. It thus provides a versatile diagnostic aid since such parts (specifically identifiable amino acid residues) may be damaged in disease processes or otherwise involved in drug transport or in toxic events affecting the body.

It is also possible to assign resonances for residues other than the N-terminus (i.e. Asp-Ala-His-Lys) of albumin in NMR spectra of intact blood plasma. For example spin systems assignable to threonine and tyrosine residues can be identified. this will enable the method to be used for a wider range of applications, i.e. for studying binding of molecules such as drugs which may not just affect the N-terminus.

Another feature of the invention is that normal single-pulsed acquisition of NMR spectra is preferred and is combined with spectral resolution enhancement which allows the resonances of interest to be identified and quantified. Particularly effective and therefore highly preferred is application of a combination of exponential and sine-bell functions to the free induction decay. For example the reader is referred to Prog. Nucl. Magn. Reson.Spectroscop. 1981,14, pp27-66. The enhancement can be supplemented with other pulse sequences which retain contributions from albumin in the free induction decay; these include spin-echo methods with short inter-pulse delays.

For further reference to suitable resolution enhancement, the reader is directed to Analyst, March 1993, vol 118, 241-244.

If the sample to be subjected to the $^1$H-NMR albumin detection method contains water there will almost certainly be signal interference from the protons in the water which can affect the accuracy of these methods. If the water cannot be removed or avoided, it is advisable to adopt known water signal suppression techniques whilst carrying out the resonance. For example when using 2-Dimension (2D) spectroscopy, the SCUBA suppression as described in J. Magn.Resn. (1986), vol 66, p379 can be utilised. For 1-Dimensional (1D) spectroscopy, the 'jump and return 1331' or pre-saturation techniques described in (1983) J. Magn. Resn., Vol 55, p283 can be used.

Alternatively it is possible to avoid interference from water protons by eliminating water from the physiological fluid sample under investigation. For example the same may be freeze dried and then re-suspended in heavy water $D_2O$.

The method can be used to detect abnormalities of albumin in diseased or other states, to monitor the course

of therapy, to determine interactions of drugs, xenobiotics and other agents, and to monitor the state of preservation of plasma or serum and other albumin-containing physiological fluids.

Table I $^{1}$H-NMR chemical shifts for Ni-albumin, Ni-albumin, Ni-albumin formed in blood plasma, and the $Ni^{2+}$ complex of the 1-24 peptide of human albumin.

| proton | | Ni-albumin | Ni-plasma | Ni-[1-24]peptide |
|---|---|---|---|---|
| Aspl | αCH | 3.60 | 3.58 | 3.58 |
| | $\beta CH_2$ | 2.45 | 2.45 | 2.43 |
| | $\beta' CH_2$ | 2.60 | 2.59 | 2.60 |
| Ala2 | αCH | 3.63 | 3.65 | 3.68 |
| | $\beta CH_3$ | 1.27 | 1.25 | 1.30 |
| His | $\epsilon$CH | 7.47 | 7.48 | 7.56 |
| | $\delta$CH | 6.75 | 6.73 | 6.94 |
| Lys4 | $\delta CH_2$ | 1.50 | 1.52 | |
| | $\epsilon CH_2$ | 2.84 | 2.87 | |

EXAMPLES:

A resolution-enhanced 500 MHz (Bruker AM500) single pulse spectrum of the aromatic region of human blood plasma is shown in Figure 1. A large number of the peaks have similar chemical shifts, intensities and line widths to those observed in spectra of defatted purified human albumin (Fig. 2) over a range of pH values. High field-shifted resonances are also observable in plasma spectra (0.5 to -0.5ppm) corresponding to those of isolated albumin, and several

peaks in the aromatic region undergo pH-dependent shifts (data not shown) similar to peaks of isolated albumin assigned to His $\epsilon$CH (C2H) imidazole ring protons. Additional small sharp peaks in the plasma spectrum are assignable to formate, and the free amino acids His, Tyr and Phe (confirmed by the spectrum of the low $M_r$ ultrafiltrate from the same plasma sample, Fig. 2).

Next we titrated $Ni^{2+}$ into plasma. Most notable was the progressive appearance of a new peak at 7.48 ppm (VI) and the disappearance of His $\epsilon$CH peaks I and II, together with a reduction in intensity of peak III, Figure 1A. In the His $\delta$CH (C4H) region, two peaks IV and V disappear, and a new peak appears at 6.73 ppm (VII). Peaks II and IV can be assigned to the $\epsilon$CH and $\delta$CH, respectively, of free His (confirmed by a standard addition). Peaks VI and VII have similar shifts to the His $\epsilon$CH and $\delta$CH peaks of Ni-albumin, Table 1. Changes in the aliphatic region of spectra of plasma on addition of $Ni^{2+}$ also show a striking resemblance to those observed for isolated albumin. Cross-peaks in 2D TOCSY spectra of plasma assignable to Aspl $\alpha/\beta$ and $\alpha/\beta'$ protons decrease in intensity, and new cross-peaks assignable to the same protons in the $Ni^{2+}$ complex appear, Figure 3. The cross-peak for Ala2 $\alpha/\beta$ protons can be obscured by lipid peaks in the spectrum of plasma but can be assigned in the $Ni^{2+}$ complex, Figure 3, Table 1. Little change was observed in the high field region of the spectrum (0.5 to -0.5 ppm) on addition of $Ni^{2+}$. No further increases in the intensities of peaks VI and VII occurred after addition of ca. 0.75 mol equiv $Ni^{2+}$ (Fig. 4), but only a general broadening of peaks attributable to the formation of paramagnetic $Ni^{2+}$ complexes.

Figure 1

500 MHz $^1H$ NMR spectra of the aromatic region of human blood plasma, before (lower) and after (upper) addition of $Ni^{2+}$ (ca. 0.5 mol equiv with respect to

albumin). The spectra have been resolution-enhanced using a combination of exponential and sine-bell functions. Assignments - albumin: I (His $\epsilon$CH), III (His3 $\epsilon$CH), V (His3 $\delta$CH); Ni-albumin: VI (His3 $\epsilon$CH), VII (His3 $\delta$CH); L-His; II ($\epsilon$CH), IV ($\delta$CH);

Figure 2

Resolution-enhanced 500 MHz $^1$H NMR spectra of the aromatic region of (a) isolated defatted human serum albumin (Sigma, cat. no. A-3782), (b) human blood plasma, and (c) the low-molecular mass (< 5 kDa) ultrafiltrate of blood plasma. Assignments: For formate, Phe phenylalanine, Tyr tyrosine, see also Fig. 1A. All samples were at pH 7.04.

Figure 3

500 MHz 2D TOCSY $^1$H NMR spectra (spin-lock time 35 ms) of human blood plasma, before (lower) and after (upper) the addition of $Ni^{2+}$ (ca. 0.5 mol equiv with respect to albumin). New peaks (labelled with an asterisk) appear which are assignable to the N-terminal amino acid residues of Ni-albumin (Aspl-Ala2-) and a Lys residue. Very similar changes are observed when isolated defatted human albumin binds $Ni^{2+}$, Table I.

Figure 4

Graphs showing the changes in peak heights (as a percentage of the initial height) of selected resonances in $^1$H NMR spectra of blood plasma at various $Ni^{2+}$: albumin mol ratios.

Figure 5 - The 2 D TOCSY spectra of human serum albumin (HSA) in $D_2O$ on a 600 mHz spectrometer where reference 3 shows the Tyr $\delta/\epsilon$ crosspeak.

Figure 6 - The 2 D TOCSY spectra of blood plasma (freeze dried and reconstituted in $D_2O$) wherein 3a indicates the same crosspeak as in Figure 5

Figure 7 - The 2 D TOCSY spectra of HSA in $D_2O$ where reference 1 shows th Thr $\beta/\gamma$ crosspeak and 2 shows the Thr $\alpha/\gamma$ crosspeak.

Figure 8 - The 2 D TOCSY spectra of blood plasma (freeze dried and reconstituted in $D_2O$) wherein 1a and 2a show the same crosspeaks as in Figure 7.

CLAIMS

1. A method of detecting albumin or one or more amino acid residues thereof, in a physiological fluid containing albumin and other proteins, and optionally free amino acids, which comprises subjecting a sample of the fluid to $^1H$ NMR spectroscopy which is single pulse or 2 dimensional spectroscopy, in which the resolution is enhanced.

2. A method as claimed in claim 1 wherein the physiological fluid comprises intact plasma.

3. A method as claimed in claim 1 wherein the physiological fluid comprises intact serum.

4. A method as claimed in any preceding claim wherein water has been substantially removed from the sample such as by freeze drying.

5. A method as claimed in any preceding claim wherein the sample is suspended in heavy water ($D_2O$).

6. A method as claimed in any preceding claim wherein at least one amino acid residue at the N-terminus of the albumin molecule is detected.

7. A method as claimed in any preceding claim further including steps to derive a quantitative determination of the albumin or amino acid residue detected with reference to a standard sample of known concentration.

8. A method as claimed in any preceding claim carried out on a high frequency NMR spectrometer of at least 500 MHz frequency.

9. A method as claimed in any preceding claim including water (proton) signal suppression.

10. A method as claimed in any preceding claim wherein the spectroscope is constructed and arranged to produce 2-dimensional spectra.

11. A method as claimed in any preceding claim wherein the resolution enhancement comprises a combination of exponential and sine-bell functions to the free induction decay.

12. A method as claimed in any preceding claim further including pulse sequence supplementation which retain albumin signal contributions.

13. A method as claimed in any preceding claim which includes use of a metal-complex forming reagent.

14. Use of the method as claimed in any preceding claim in the monitoring of albumin within a physiological fluid such as human blood plasma or human blood sera.

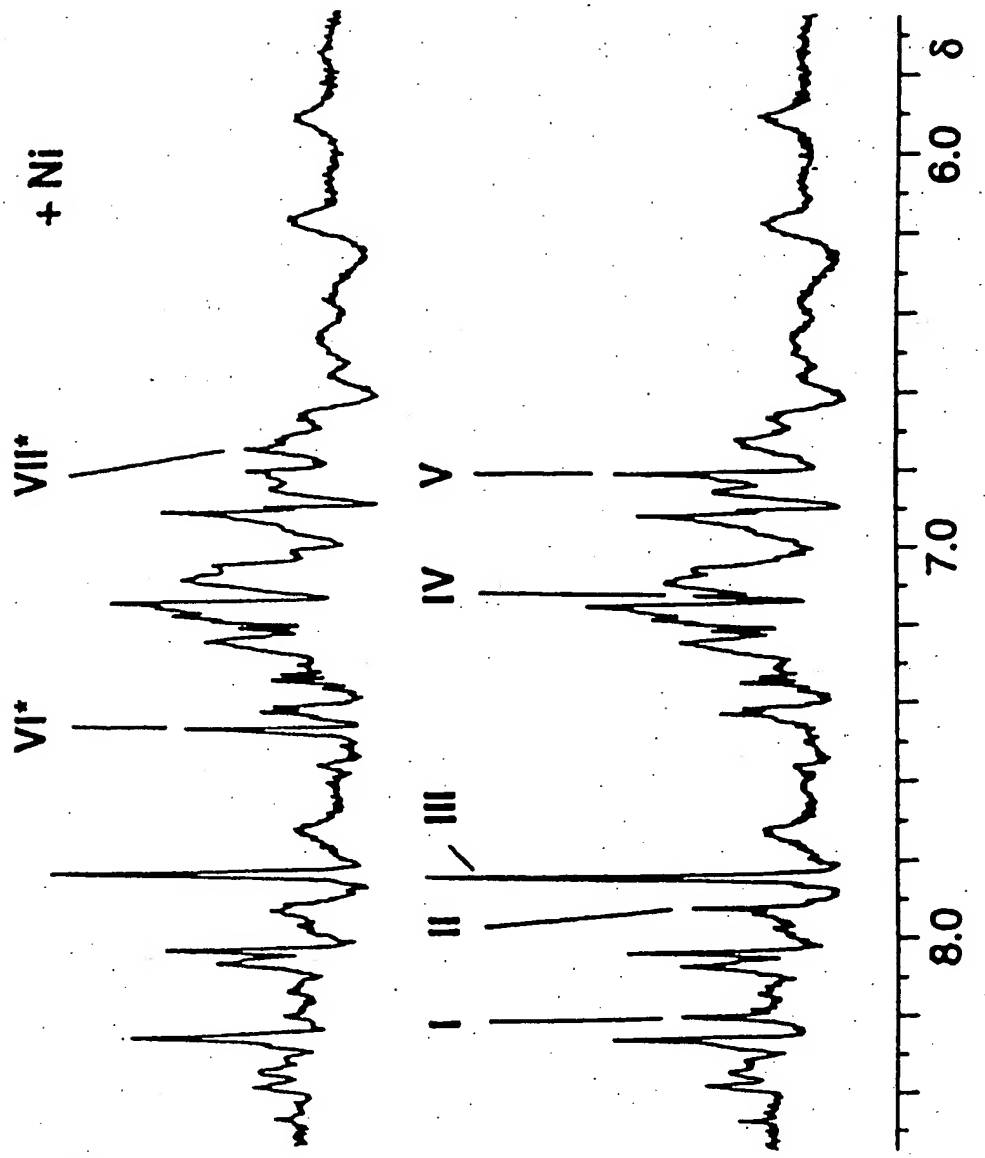
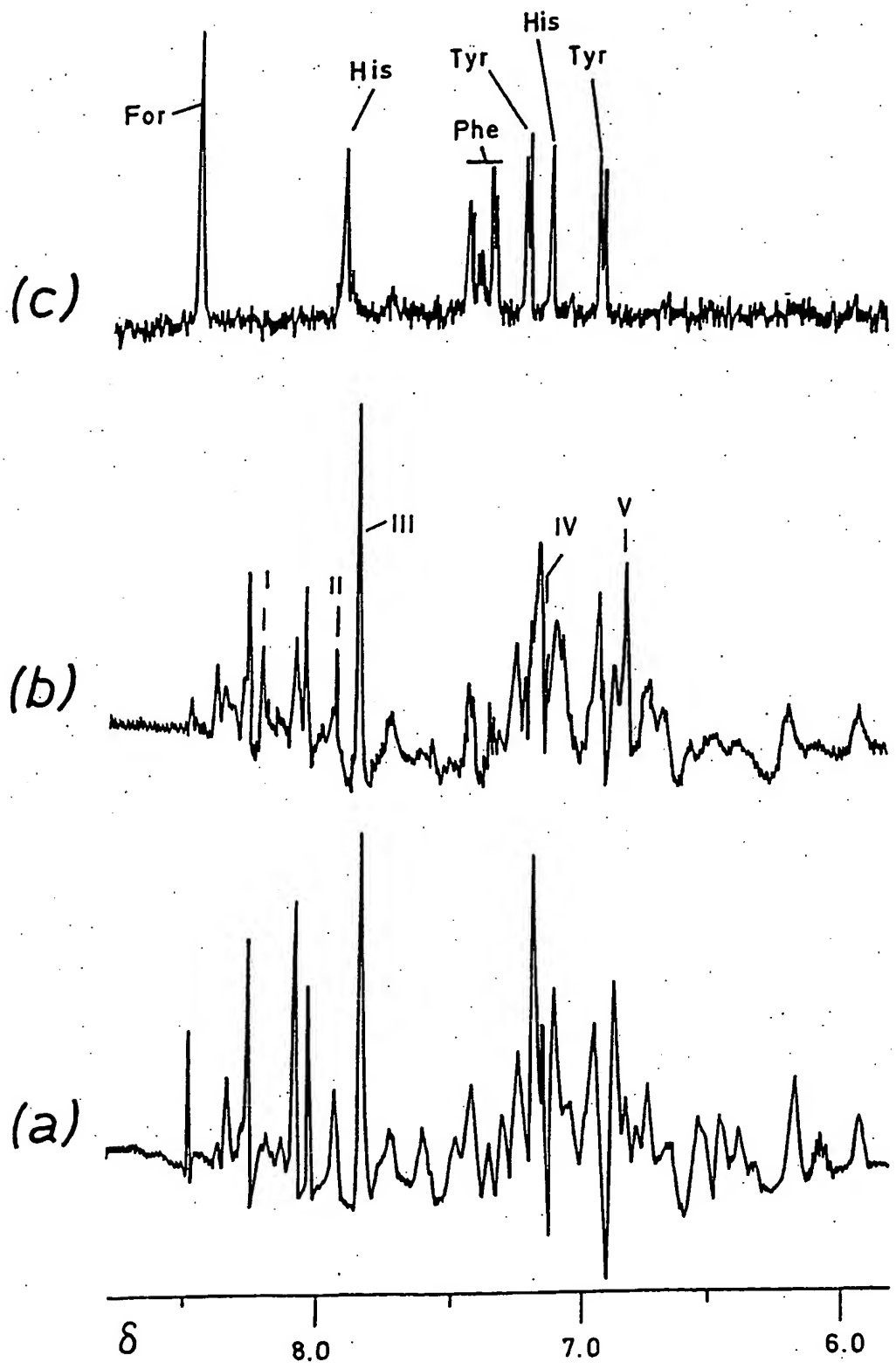



FIG.1

FIG. 2



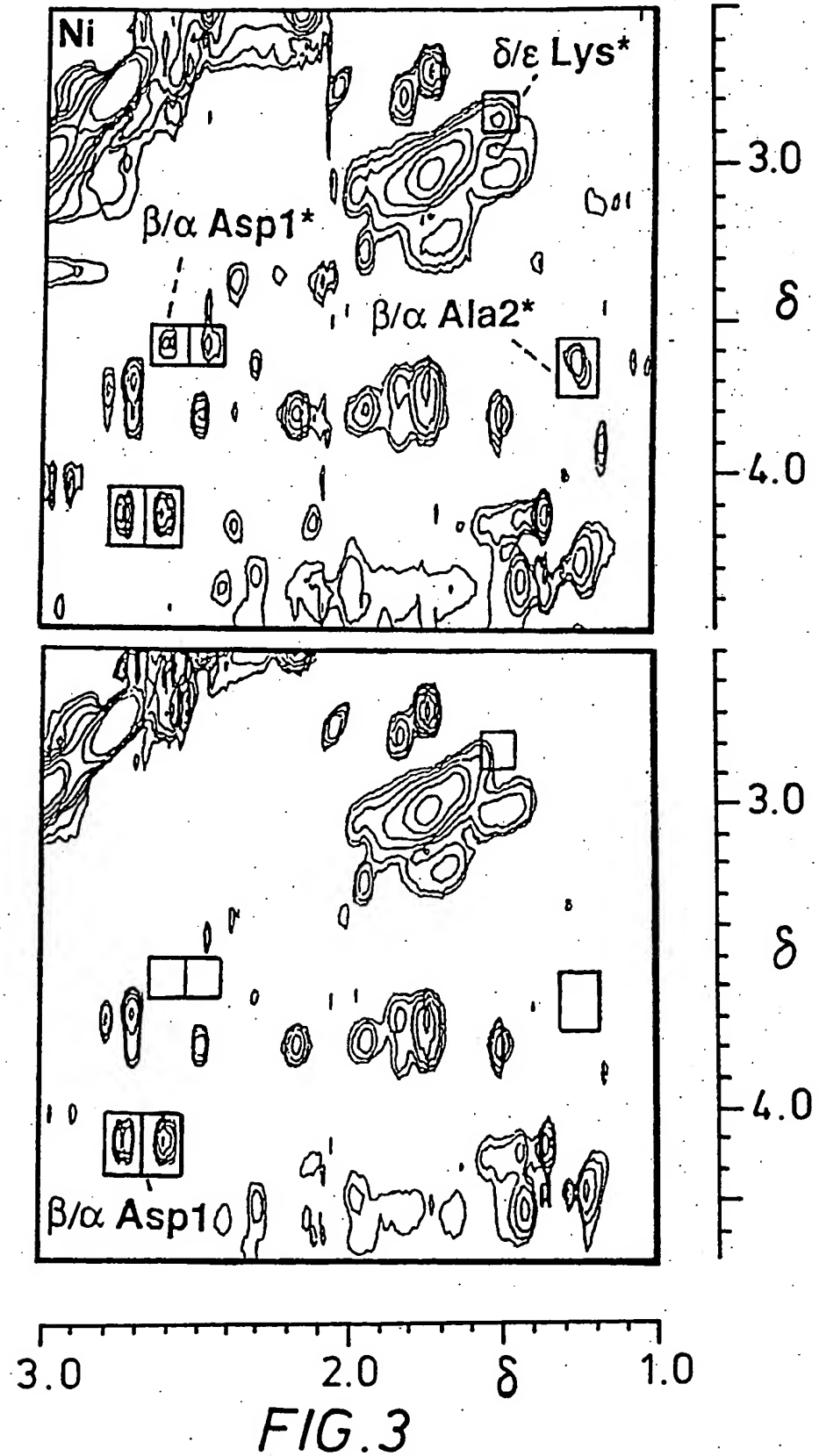



FIG.3

FIG. 4

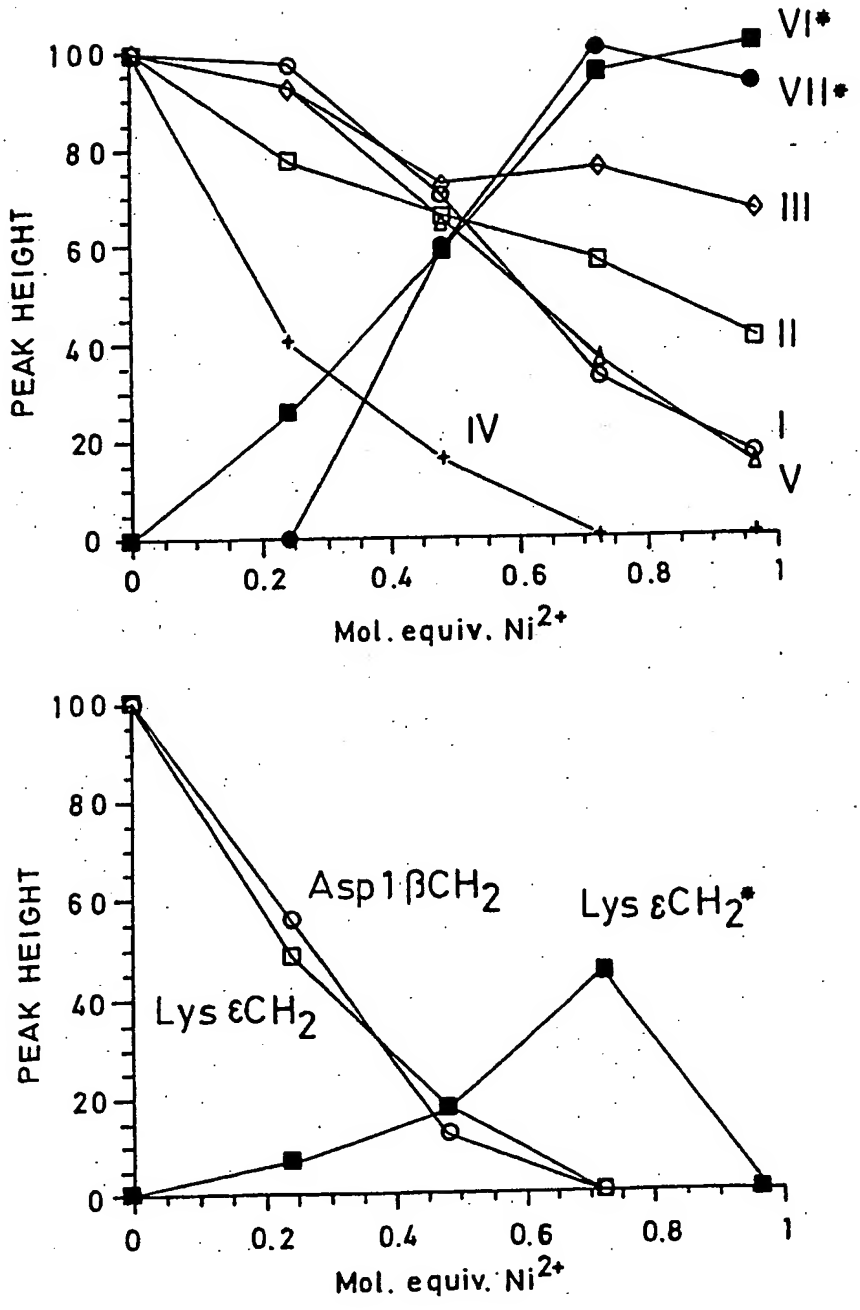

VI*
VII*
III
II
I
V
IV
PEAK HEIGHT
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
Mol. equiv. $Ni^{2+}$
Asp1 βCH2
Lys εCH2*
Lys εCH2
PEAK HEIGHT
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
Mol. equiv. $Ni^{2+}$

FIG.5

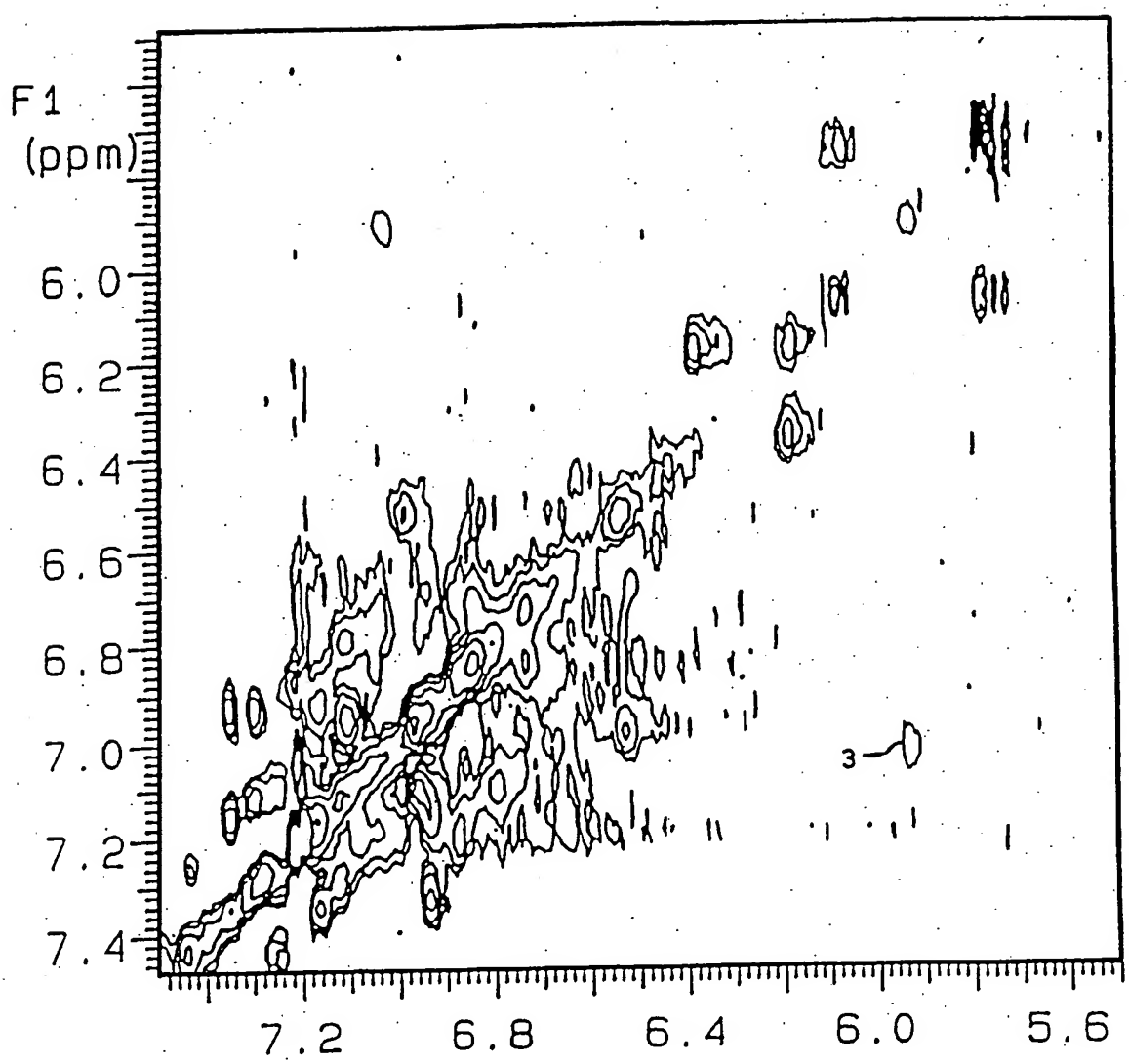
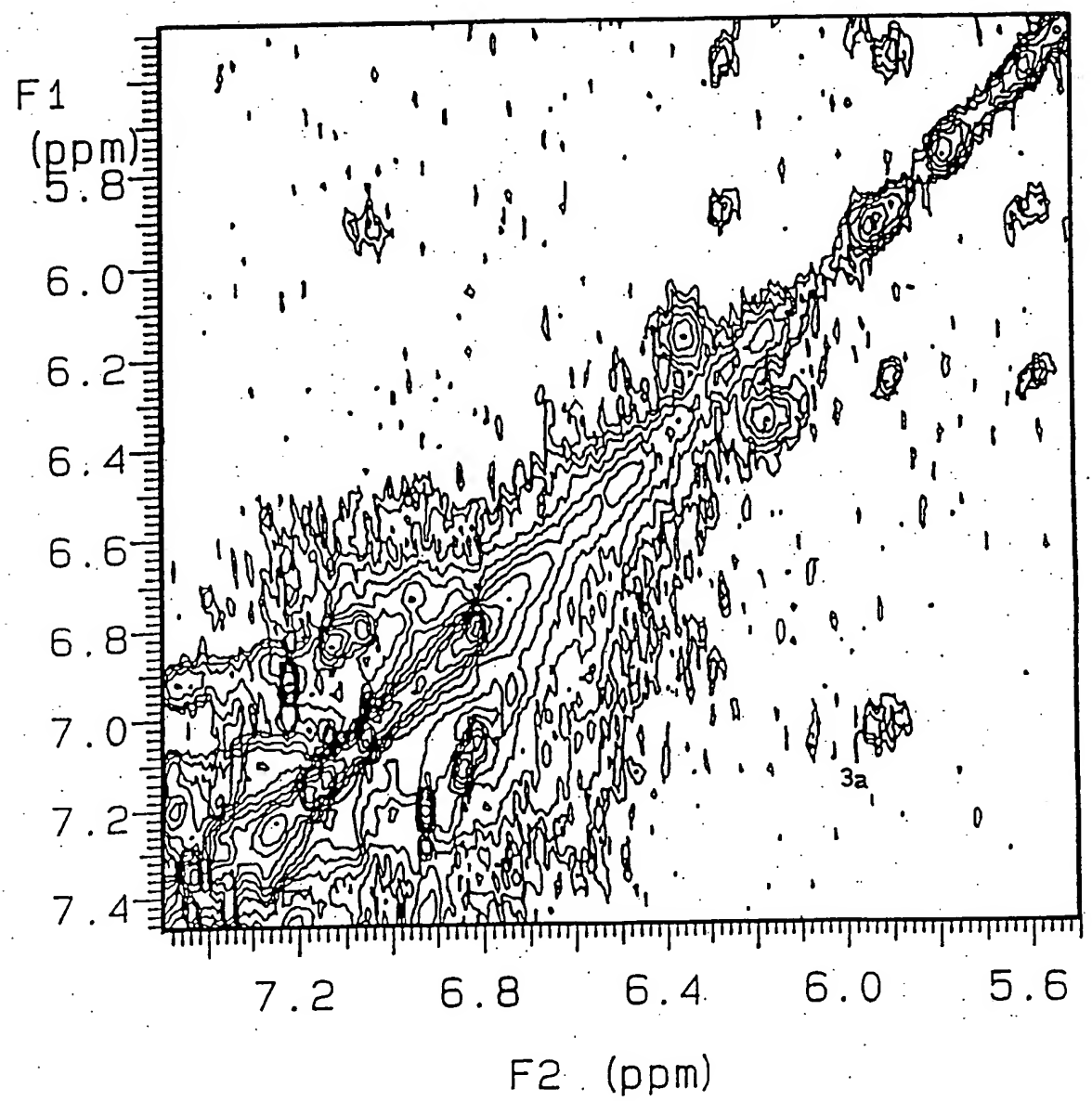


FIG.6



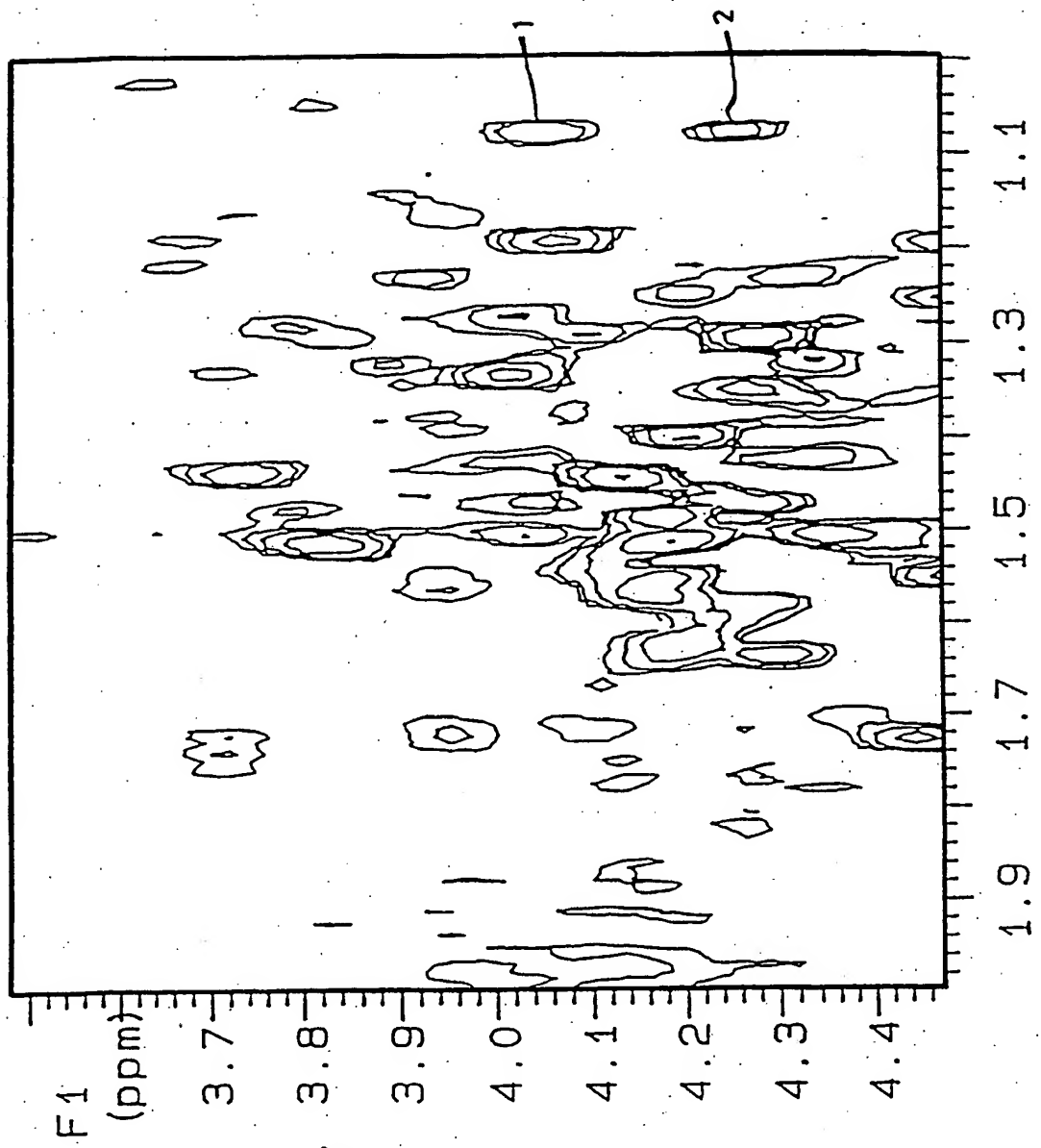



FIG. 7

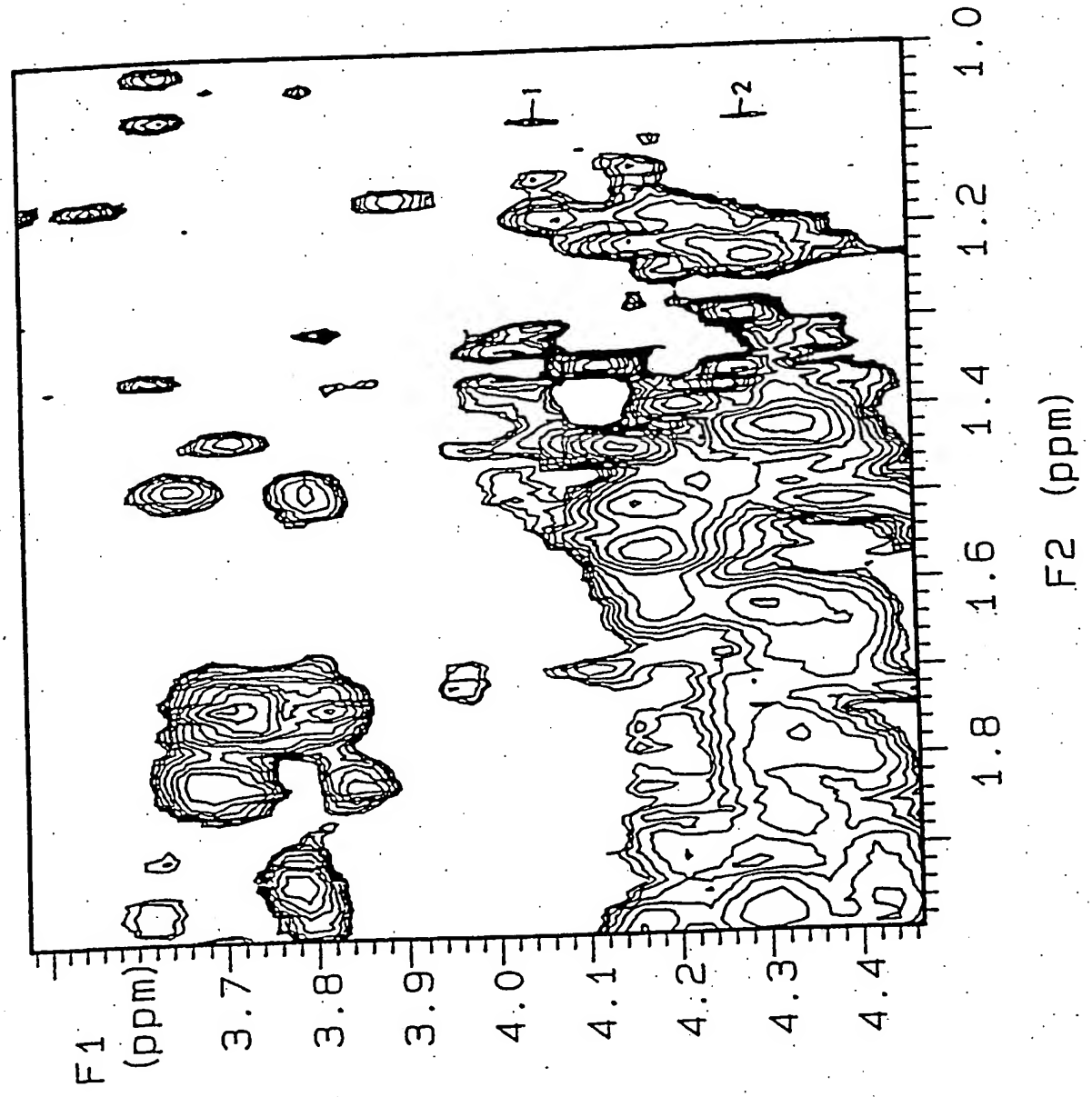



FIG. 8

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/00425

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01R33/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EUROPEAN JOURNAL OF BIOCHEMISTRY, vol.205, 1992 pages 631 - 643 P.J. SADLER, A. TUCKER: 'Proton NMR studies of bovine serum albumin' cited in the application see pages 631-632, paragraphs "Introduction" and "Experimental Procedures" --- | 1,3-6, 8-12,14 |
| X | FEBS LETTERS, vol.219, no.1, July 1987, AMSTERDAM, NL pages 239 - 243 J.D. BELL ET AL.: '1H NMR studies of human blood plasma' | 1,2,4,6, 8,9,13 |
| A | see pages 239-241, paragraphs 1-3 --- -/-- | 3,7,11 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 19 May 1994 | 15.06.94 |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Authorized officer<br>Volmer, W |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/00425

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP,A,0 234 524 (THE BETH ISRAEL HOSPITAL ASSOCIATION) 2 September 1987 | 1-3,7,9, 13 |
| A | see page 2, line 57 - page 4, line 46 | 8 |
| A | WO,A,92 01946 (BRUKER ANALYTISCHE MESSTECHNIK GMBH) 6 February 1992<br>see page 1, paragraph 1<br>see page 6, paragraph 4 - page 7, paragraph 1<br>see page 9, paragraph 2 -paragraph 3<br>see page 13, paragraph 2 - page 14, paragraph 4<br>see page 15, paragraph 4 - page 16, paragraph 2<br>see page 20, paragraph 1 - page 21, paragraph 4<br>see page 24, paragraph 1 -paragraph 2<br>see page 26, paragraph 2 - page 27, paragraph 4<br>see claims 4,5,9,12 | 1,4,5, 7-10,12 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/GB 94/00425

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| EP-A-0234524 | 02-09-87 | AU-B- | 600931 | 30-08-90 |
| | | AU-A- | 6918987 | 27-08-87 |
| | | DE-D- | 3788812 | 03-03-94 |
| | | DE-T- | 3788812 | 05-05-94 |
| | | JP-A- | 62276459 | 01-12-87 |
| | | US-A- | 4918021 | 17-04-90 |
| | | US-A- | 4912050 | 27-03-90 |
| | | US-A- | 5213101 | 25-05-93 |
| | | US-A- | 5261405 | 16-11-93 |
| WO-A-9201946 | 06-02-92 | EP-A- | 0493386 | 08-07-92 |